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THE ROLE OF AMOEBOCYTES IN THE REGENERATION OF SHELL IN THE LAND PULMONATE, *EUPLECTA INDICA* (PFIEFFER)

S. P. KAPUR AND A. SEN GUPTA

Department of Zoology, Panjab University, Chandigarh, India

The presence of amoebocytes at the site of shell regeneration in gastropods has been observed by several workers. But opinions and observations differ as to the manner in which the amoebocytes participate in the repair process. Durning (1957) states that in *Helix aspersa* amoebocytes appear along cracks in the regenerating membrane due to irritation and he dismisses them as unconnected to shell repair. McGee-Russell (1954) also noted in regenerating snails the presence of some cells in the extrapallial fluid that originated from mantle epithelium but he did not assign them any role in the repair process. Abolins-Krogis (1963, 1968) opines that during shell repair, amoebocytes transfer large amounts of repairing materials like proteins and calcium from the yellow body cells and digestive gland cells to the actual site of shell repair, liberate it there and withdraw from the site. But some of the cells become accidentally trapped within the calcifying plates of the shell. To complicate matters further, studies of Kapur and Gibson (1967) on the normal development of shell in *Helisoma duryi eudiscus* point to the appearance of amoebocytes on the ostracum of the shell in the juvenile snails and that later these form multinucleate organic plates. These have been suggested to be directly involved in the calcification of the adult shell.

With such divergent views on the role of amoebocytes in the repairing as well as normal shells, the controversy is wide open for more studies. Also since studies on shell repair in gastropods have almost exclusively been on a single land snail, *Helix*, it was decided that new investigations should be carried out in other genera of land pulmonates. The present communication deals with the regeneration of shell in *Euplecta indica* (Pfeiffer), a terrestrial pulmonate from Chandigarh, India.

Materials and Methods

Specimens of *Euplecta indica* were collected from a garden in Chandigarh. They were maintained in a terrarium at 21° C and were fed on cabbage leaves. Seven groups of thirty snails each were used in this study. A hole, about 3 to 4 mm in diameter, was made in the shell of each snail, some distance behind the shell aperture. It was then covered with a piece of glass coverslip, using warm paraffin wax for pasting. The snails deposited the regenerating shell material on the undersurface of the coverslips. At ten different intervals, ranging from 1 hour to 108 hours, the coverslip bearing the regenerate material was removed from the shell, placed in the required fixative and processed accordingly. Regenerate materials obtained thus at different intervals from each group of 30 snails were stained with the following techniques: group 1, haematoxylin and eosin; group 2, toluidine blue;

group 3, alcian blue-Delafield haematoxylin; group 4, mercuric bromophenol blue (Hg-BPB); group 5, periodic acid-Schiff (PAS) with and without diastase digestion; group 6, Sudan black B and group 7, Feulgen test. Regenerate samples from 1 to 4 and 7 were fixed in 10% neutral formaline, from group 5 in Bouin's fluid and from group 6 in formol-calcium. Some neutral formalin-fixed samples of shell regenerate were tested for the presence of calcium, using the von Kossa technique. In group 3, Delafield haematoxylin was used as a nuclear counterstain instead of the recommended neutral red because batches of this dye then available were not compatible with the alcian blue technique. All the histochemical techniques were carried out as described by Pearse (1961). The regenerate-bearing pieces of coverslip after staining, dehydration and clearing were mounted as whole mounts in Canada balsam, with the regenerate-bearing surface facing upwards. Samples from group 6 were similarly mounted in glycerine jelly.

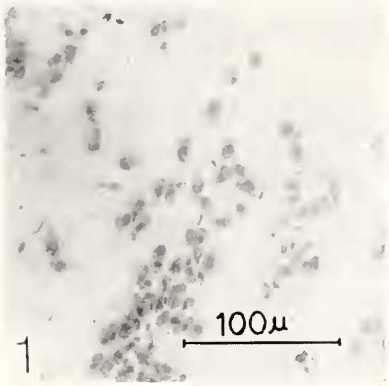
It was observed during this study that given everything equal, including size of animals, their time of collection and feeding, the rate of shell repair varied between individuals. The timings given are not rigid invariables, but represent the average conditions seen in the greatest number of individuals in all the groups used in this study.

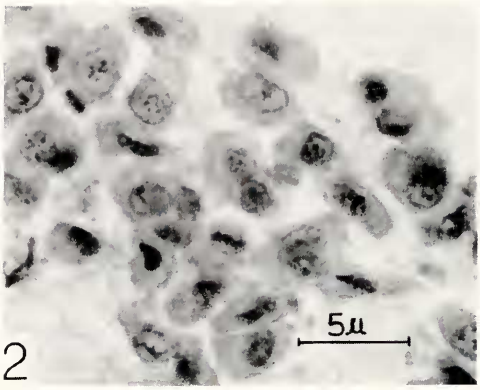
Observations

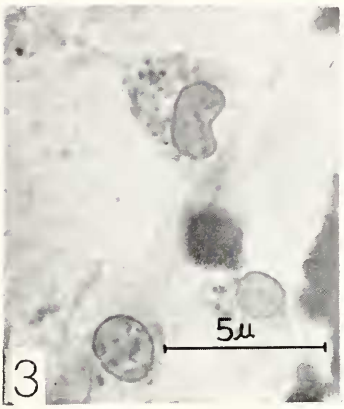
The process of shell repair is initiated by the deposition of a glistening, translucent regeneration membrane, one hour after shell damage. This membrane stains uniformly blue with haematoxylin, is uniformly PAS positive with and without diastase digestion, is slightly metachromatic in toluidine blue, stains bluish-green in alcian blue, gives a positive test with Hg-BPB and is also weakly sudanophil. No localization of staining was observed by any of the above techniques, as the regeneration membrane is homogeneous and structureless. The above mentioned tests show the presence of neutral mucopolysaccharides, glycoproteins, mucoproteins, acid mucopolysaccharides, proteins and small amounts of lipids.

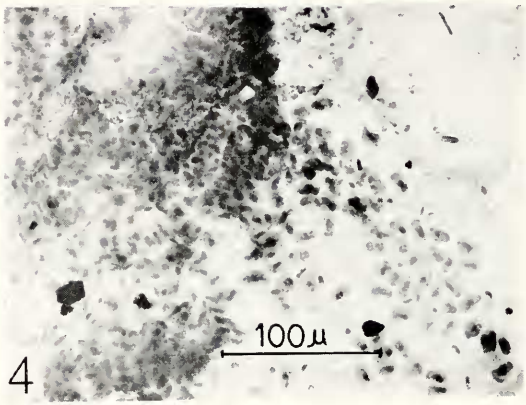
One hour after the start of regeneration, amoebocytes suddenly make their appearance on the regenerating membrane, continue to increase in number until at six hours their number reaches the highest peak (Fig. 1). Two types of amoebocytes were observed and we designate them as the a-type and the b-type. Nuclei of a-type cells are deeply basophilic with a compact coarsely granular meshwork of chromatin. Nuclei of b-type cells are vesicular, with scattered chromatin granules interlinked by fine fibrils. The nucleo-cytoplasmic ratio of the two kinds of cells also differs: nuclei of a-type cells occupy a third or a quarter of the total cell volume whereas nuclei of the b-type cells occupy more than half the extent of the cell (Fig. 2). Further, the a-type amoebocytes are about half the size of the b-type amoebocytes, but occasionally acquire the full size of the b-type amoebocytes. Occasionally the nuclei of some a-type amoebocytes possess U, V, J or dumbbell shapes. Possibly the nuclei of these cells are undergoing necrosis (Fig. 6).

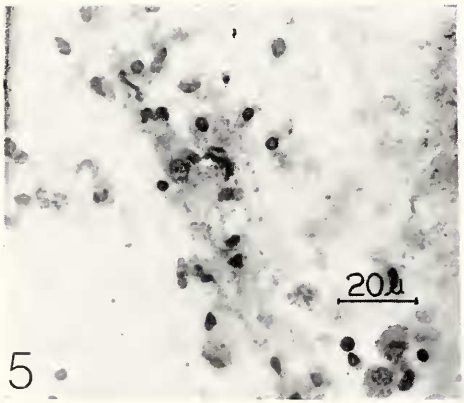
The nuclei of both types of cells showed the usual basophilia with haematoxylin and toluidine blue. Besides, the chromatin granules in both types of nuclei show metachromasia with toluidine blue and also stain positively with alcian blue. The general cytoplasm of both cells is eosinophilic toluidine blue positive and alcian blue positive. The cytoplasmic granules of the a-type amoebocytes show metachromasia

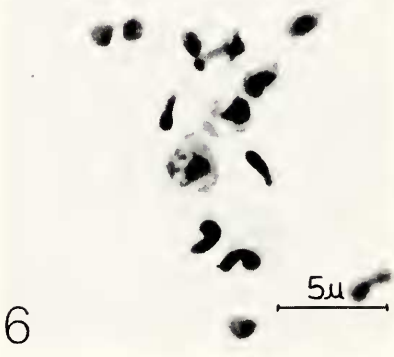












FIGURES 1–6.

with toluidine blue. Calcium is present in the cytoplasm of both types of amoebocytes in the form of granules (Fig. 3). Nuclei of both cell-types give a positive test with Hg-BPB but the nucleus of the a-type does so more intensely, while the nucleoplasm of the b-type remains unstained. The cytoplasm of both cell-types is also positive to Hg-BPB but in the b-type cells, a narrow perinuclear zone remains unstained. Nuclei of both cell-types are negative to Sudan black B staining but their cytoplasm is diffusely positive. Some sudanophilic granules are present in the perinuclear zone which as stated above, gives no response to Hg-BPB staining. Nuclei of both cell-types are Feulgen positive.

The preceding observations relate to the amoebocytes that have just arrived at the regeneration site. But soon, about an hour after their arrival, the amoebocytes cluster and start losing their identity (Fig. 4). It appears that their outer cell membranes rupture and their cytoplasm precipitates, leaving their nuclei in a free, denuded state (Fig. 5). Such patches of precipitated cell material exhibit the same staining reactions as in the intact amoebocytes. They give positive results for neutral mucopolysaccharides, glycoproteins, mucoproteins, acid mucopolysaccharides, proteins and lipids. The free nuclei are seen as long as 83 hours after the precipitation of the amoebocytes. However, gradually, these nuclei become pycnotic, diminish in size and finally disintegrate. Such disintegrated nuclear material along with the precipitated cytoplasm is still Feulgen positive. The a-type cells with U, V, J and dumbbell-shaped nuclei also behave in the same manner (Fig. 6).

The site of repair now has a regeneration membrane bearing large numbers of intact amoebocytes and also patches of precipitated cells which had arrived earlier. In addition, numerous fine tracts of acid mucopolysaccharides are observed. These are possibly derived from the secretion products present in the extrapallial fluid which is constantly bathing the regenerating material.

The first indication of the initiation of the calcifying process of the regenerate is given by the appearance of small crystals of calcium carbonate on top of or along the precipitated cytoplasm of the amoebocytes and the fine tracts of acid mucopolysaccharides. These crystals are in spheritic double-fan shaped arrays (Fig. 7). Thus the materials contributed by the amoebocytes serve as the calcifying substratum. As crystallization proceeds, a steady decline in the metachromasia of the organic substratum is noted. The regeneration plate thus comes to have several calcifying tracts showing crystallization of calcium carbonate, coincidental with the tracts of cellular precipitation. The process of arrival of amoebocytes and their precipitation is a continued one, occurring simultaneously with crystal formation from this point onwards. Thus the precipitation of the amoebocytes contributes to

FIGURE 1. Two-hour regenerate: amoebocytes on the regeneration membrane; toluidine blue.

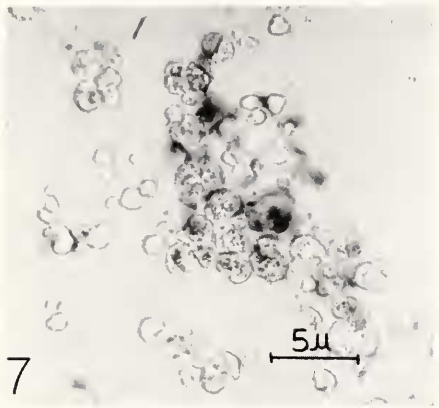
FIGURE 2. Six-hour regenerate: a- and b-type amoebocytes. Note the difference in the nuclei of the two cell types; toluidine blue.

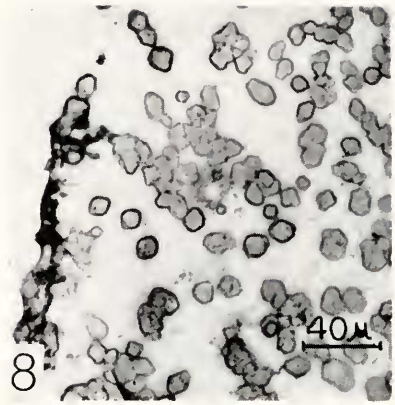
FIGURE 3. Four-hour regenerate: note the calcium granules in the cytoplasm of the amoebocytes; von Kossa-neutral red.

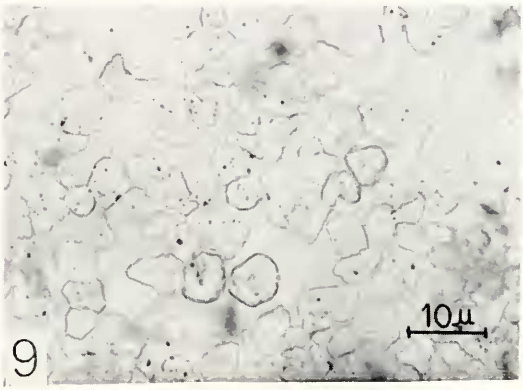
FIGURE 4. Six-hour regenerate: intact cells at right, precipitated cells on the left of the picture; toluidine blue.

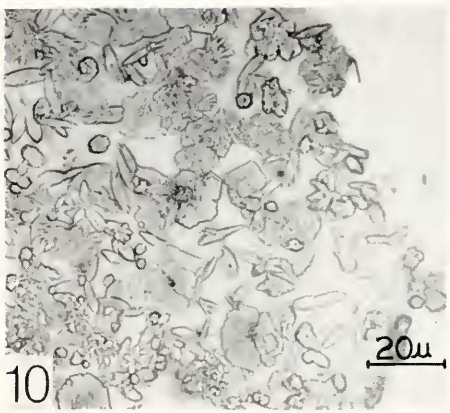
FIGURE 5. Six-hour regenerate: free nuclei lying in the precipitated cytoplasm; alcian blue-Delafield haematoxylin.

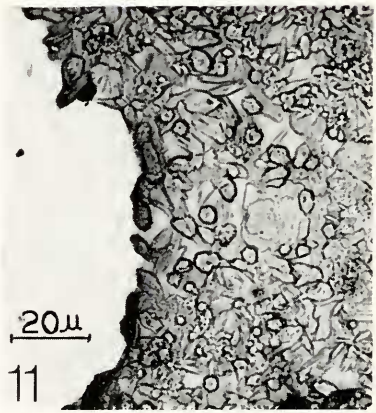
FIGURE 6. Six-hour regenerate: free pycnotic nuclei of various shapes; one intact b-type amoebocyte can be seen; toluidine blue.











FIGURES 7–11.

the formation of the organic ostracum upon which the growth of the mineral fraction of the shell proceeds. The growing calcium carbonate crystals soon acquire a tabular form (Fig. 8).

As these crystals grow larger, they meet adjacent growing tabulae at their peripheries and coalesce with them (Fig. 9). Small acicular crystals appear in parallel and in spheritic aggregates in the spaces between such intergrowing tabulae (Fig. 10). A second layer of crystals is initiated on top of the first layer even before the latter is completed. Thus the regenerate grows in extent as well as in thickness at the same time.

At 24 hours the regenerate consists of an almost complete sheet of tabulae, with most of the intervening spaces filled up by parallel and spheritic aggregates of crystals (Fig. 11). New batches of amoebocytes continue to arrive and precipitate, the only difference now being that whereas the first batches arrived and precipitated on the regeneration membrane, the later arrivals precipitate upon the crystalline layers. Successive layers of crystals are laid down until in about a week's time the regenerate approaches the original shell in thickness.

In surface view the regenerate appears as a thick conglomerate of crystals. It actually consists of several indistinctly separated crystalline sheets, each of which may be seen in a different plane of focus at the edges.

The fully regenerated shell is intimately fused with the original shell and cannot be easily separated from it. The regenerate lacks a periostracum, which is present in the normal shell. It also differs from the latter in its random arrangement of crystals and in its partly cellular origin, of which the normal shell presents no evidence.

Discussion

It has been observed in this study that within an hour of shell damage, a homogeneous, thin, translucent membrane appears over the damage area of the shell. This has probably come about by the precipitation of secreted organic substances present in the extra-pallial fluid. To this extent shell regeneration is a physico-chemical process. But we cannot agree with workers like Manigault (1933) and McGee-Russell (1954) on *Helix* that the entire regeneration of shell is brought about in this manner. On the other hand observations of Wagge (1951) also on *Helix* that the regeneration membrane is formed exclusively by the amoebocytes which arrive at the site to deliver proteins and calcium are not tenable here. What we find is that once the precipitated membrane is formed at the beginning of regeneration, it acts as a scaffolding for the amoebocytes to perform their role in regeneration.

Figure 7. Seven-hour regenerate: initial double-fan shaped and spheritic crystal aggregates in association with the cellular precipitate and two free pycnotic nuclei; toluidine blue.

Figure 8. Nine-hour regenerate: growing tabloid crystals on the organic ostracum.

Figure 9. Ten-hour regenerate: intergrowth of tabulae. Note coalescence at the margins of the crystals.

Figure 10. Twelve-hour regenerate: parallel and spheritic crystal aggregates appearing in the spaces between the tubulae.

Figure 11. Twenty-four-hour regenerate: note that the spaces between the tabulae are nearly filled by the parallel and spheritic aggregates of crystals.

Further it is observed that an hour after the start of shell regeneration, large numbers of amoebocytes arrive at the newly formed regeneration membrane. But unlike observations of Wagge (1951) it is found that these cells do not merely deliver their payloads and then withdraw from the membrane. Instead, it appears that the amoebocytes lose their identity, their outer cell membranes become disorganized and their cytoplasm precipitates, leaving free nuclei. In fact the amoebocytes arrive in such large numbers that the original regeneration membrane is no longer seen. Histochemical tests on the intact and disorganized cells reveal the presence of large amounts of protein, PAS positive mucopolysaccharides and acid mucopolysaccharides. These substances are necessary components of most calcifying matrices in animals. Besides, calcium granules were also detected in the cytoplasm of the amoebocytes. These findings are in agreement with those of Abolins-Krogis on *Helix* (1960, 1963 and 1968) that amoebocytes carry histochemical substances necessary for calcification from different organs of the body to the site of regeneration. We further find that not only are these substances transferred to the regeneration site, but also that the cytoplasm of the amoebocytes contributes predominantly to the formation of the organic ostracum on which the calcification processes are initiated.

The calcification of the regenerate is initiated by the appearance of small double-fan shaped spheritic aggregates of crystals. These initial crystals have also been observed by Abolins-Krogis (1968) in her studies on *Helix,* although she invests their structure with a large amount of organic content.

The metachromasia noticed prior to the beginning of crystallization is seen to recede in the later stages of calcification. This may be indicative of the binding of calcium by acid radicles on the acid mucopolysaccharide molecules (Tanaka and Hatano, 1955; Horiguchi, 1956; Kado, 1960) which are in association with the protein matrix. As the tabloid crystals grow large, they fuse with adjacent crystals and tend to form a continuous sheet. New batches of amoebocytes continue to arrive and settle over the crystalline sheets and thin films of organic material which is probably continually precipitated from the extrapallial fluid. Soon the amoebocytes become disorganized, spill over their contents and start calcification of another crystalline sheet once again.

Although no cellular involvement is noticeable in the structure of the adult shell of *Euplecta,* the involvement of amoebocytes in the calcification of developing and adult shell is known from the studies of Kapur and Gibson (1967) and Kapur and Bansal (in preparation). In fact, Kapur and Gibson found precipitation of the newly arrived amoebocytes on the protoconch and subsequent initiation of calcification in *Helisoma duryi eudiscus.* The disruption of amoebocytes at the site of shell regeneration reminds us of a rather similar behavior of leucocytes in wound healing in vertebrates.

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Summary

An hour after the damage of the shell in *Euplecta indica* (Pfieffer) a thin, structureless, basophilic regeneration membrane appears. Thereafter, a large number of amoebocytes precipitate on this membrane. Soon these break up and contribute their cytoplasmic contents to the regenerate. This is positive for glycoproteins, mucoproteins, neutral mucopolysaccharides, acid mucopolysaccharides, proteins, calcium and some lipids. Also, tracts of acid mucopolysaccharides precipitate from the extrapallial fluid and are deposited on the regenerate.

Calcification is initiated by the appearance of seed crystals of calcium carbonate on top of or along areas of cellular precipitation. This leads to a progressive decrease in metachromasia. The crystals grow into large tabular forms, coalesce with the adjacent tabulae and form a calcified sheet of regenerated material. The spaces between the tabulae are later packed by small crystal aggregates of various shapes. More and more amoebocytes continue to arrive, settle on the calcified sheets along with precipitated organic matter from the extrapallial fluid, break up and repeat the calcifying process until a regenerate of the same thickness as the normal shell is formed.

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